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Identification of a 59 bp enhancer located at the first exon/intron boundary of the human O^6-methylguanine DNA methyltransferase gene

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ABSTRACT

The DNA repair enzyme, O^6-methylguanine DNA methyltransferase (MGMT) is responsible for repair of damage induced by alkylating agents that produce adducts at O^6-guanine in DNA. Although the MGMT gene promoter has housekeeping gene promoter characteristics, unlike these genes which are expressed at a constant level, MGMT transcriptional activity varies between cell types. During an attempt to identify regions of the MGMT regulatory sequence sensitive to variations in transcription factors between cell types, we have identified a 59 bp enhancer which is required for efficient MGMT promoter function. This fragment produced increased transcriptional activity in reporter gene constructs containing either the MGMT or UMP-synthase promoter when transfected into either of two cell lines; it seems therefore that this enhancer may interact with relatively common trans-acting factors. Functional activity is only detected when the enhancer is in 'cis' with respect to the promoter, suggesting that complexes are formed between proteins bound to the enhancer and promoter sequences. We propose that the enhancer-binding protein may be a novel transcription factor since there are no obvious consensus sequences within the 59 bp sequence for known DNA-binding proteins.

INTRODUCTION

O^6-methylguanine DNA methyltransferase (MGMT; E.C.2.1.1.63) is a DNA repair enzyme which specifically removes O^6-alkylguanine and O^4-alkylthymine adducts generated by agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and N-methyl-N'-N-nitro-N-nitrosoguanidine (MNNG) (1,2). This repair process is error free because the adduct is cleaved directly from the base without affecting the integrity of DNA structure (3,4). In contrast, other repair processes such as excision repair involve removal and replacement of several nucleotides that can result in errors in the DNA sequence (5). All normal tissues and cells express MGMT and are termed Mer$^+$ (6). However, ~20% of tumor cell lines and ~50% of SV40-transformed cell lines are deficient in MGMT expression; these are termed Mer$^-$ (7).

Mer$^-$ cells contain the MGMT gene with no gross rearrangements or deletions (8,9,10), however, MGMT mRNA is barely detectable (10) or undetectable, (8,9,11,12) suggesting that expression is inhibited at the level of transcription. Lack of MGMT expression cannot be accounted for by transcription factor deficiency because in reporter gene assays the MGMT promoter functions efficiently following transfection into Mer$^-$ cells (12,13,14). Cytosine methylation of 5' gene sequences (12,15,16) and sequences within the body of the gene (14,17,18) has been implicated in the regulation of MGMT expression.

In general, transcription is controlled by the interaction of numerous trans-acting proteins which may bind either to specific DNA sequences or to other proteins (19–21). Transcription efficiency is dependent both on the types and numbers of binding sites in a gene and on the spectrum and amounts of binding proteins expressed within a cell. Whereas there are some transcription factors (eg. proteins found in the transcription initiation complex: TFIID, TFIIE, TFIIF, etc. (22–24)) that are required for transcription by almost all promoters, others are cell-type and promoter specific, resulting in controlled expression of specific genes in the appropriate cells (25–27).

The constitutive level of MGMT in Mer$^+$ cell lines and tissues varies widely (6,28) suggesting that regulatory proteins are present in different amounts or cell-type specific factors are involved in controlling transcription. The MGMT promoter which has been identified as a 1.2 kb region of the gene (positioned −954 to +203) has characteristics of a housekeeping gene promoter in that it lacks a TATA box and is extremely CpG rich (29). Several known transcription factor binding sites occur within the promoter sequence, including ten sites recognized by Sp1 protein (13,29). It has been suggested by Pugh and Tjian (1990) (30) that Sp1 is required for transcription initiation in

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housekeeping gene promoters. Thus, Sp1 protein may be required for the initiation of MGMT transcription, however, it is unlikely that variable levels of this transcription factor account for the range of MGMT expression in Mer$^+$ cells (13).

Our current goal is to understand the regulation of MGMT gene expression in Mer$^+$ cells and our approach is to identify regions of 5′ MGMT gene sequences which are sensitive to trans-acting factor differences between cell types. Previously, we demonstrated that sequential deletion from the 5′ end of the MGMT promoter resulted in a gradual reduction of activity in a reporter gene assay (29), suggesting that the majority of the sequences contained within this area are required for efficient MGMT gene expression. In this paper we describe a 59bp DNA sequence located at the first exon/intron boundary which acts as an enhancer that is also required for efficient function of the MGMT promoter.

MATERIALS AND METHODS

Reporter gene plasmids

MGMT promoter sequences were ligated into p0CAT1 (31), a plasmid containing a promoterless chloramphenicol acetyltransferase (CAT) gene. The 869bp UMP-synthase promoter in pCATbasic (Promega, Madison, WI), was made available to us by Dr Parker Suttle, St Jude Children's Research Hospital (SJCRH). The pSV-β-Gal plasmid (Promega), which expresses β-galactosidase under the control of the SV40 promoter, was cotransfected with each of the other plasmids to control for transfection efficiency.

Transient transfections

Plasmids were transfected into cells by electroporation using a Bio-Rad Gene Pulser apparatus (Richmond, CA) at 960μF and 220V (32). CAT plasmid DNA was cotransfected with 5μg pSV-β-Gal DNA into 1×10^7 cells per experiment. CCRF-CEM cells were grown in Eagle's minimal essential medium (Life Technologies, Inc., Gaithersberg, MD) containing 10% newborn calf serum (Sigma, St Louis, MO). A204 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum. Both cell lines were grown in an atmosphere of 95% air and 5% CO_2. CCRF-CEM and A204 cell lines are both Mer$^+$ and express relatively high levels of MGMT (1−2 pmoles/mg protein).

Enzyme assays

CAT and β-galactosidase activities were measured in crude cell extracts 48 h after transfection. CAT activity was measured by a two-phase liquid scintillation counting assay using [^{3}H]-acetyl coenzyme A (Amersham, Arlington Heights, IL) as a substrate (33). β-galactosidase was assayed spectrophotometrically with chlorophenol red β-D-galactopyranoside (Boehringer-Mannheim, Indianapolis, IN) as the substrate (34). CAT activity was calculated as cpm of [^{3}H]-acetylchloramphenicol/hour per mg of protein, divided by the β-galactosidase activity to correct for differences in transfection frequency. The value for the promoterless control, either p0CAT1 or pCATbasic, was subtracted from this number.

Band mobility shift assay

The 59bp MGMT sequence from position +144 to +202, with respect to the transcription start site, was ^{32}P-labeled by the addition of ^{32}P-dCTP, other nucleotides and *Escherichia coli* DNA polymerase I Klenow fragment (Promega). CCRF-CEM nuclear extract, prepared as described by Zerivitz and Akusjarvi (35) was preincubated on ice in DNA binding buffer (20mM Tris−HCl, pH 8.0, 5mM $MgCl_2$, 0.5mM DTT, 0.1mM EDTA, 10% glycerol, 100mM KCl, 0.5mM $CaCl_2$) for 15 minutes. The binding reaction was initiated by the addition of the ^{32}P-labeled 59bp probe (5×10^5cpm) and appropriate competing DNA fragments were added at this time. After incubation at room temperature for 20 minutes, loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll Type 400 in water) was added to each sample immediately before applying to a 6%, non-denaturing polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed.

RESULTS

Identification of an enhancer sequence

Figure 1 shows the results of MGMT reporter gene assays after transient transfection into the human T lymphoblast cell line, CCRF-CEM. Removal of 184bp from the 3′ end of the MGMT promoter region up to the XbaI site reduced promoter activity by 98% (Construct 2, Fig. 1). This observation was surprising because the transcription initiation site and 20bp of the first exon remained intact. Furthermore, whereas deletion of 810bp at the 5′ end to generate a 347bp (FspI/SstI) fragment resulted in loss of 71% of the promoter activity (Construct 3, Fig. 1), removal of only another 59bp (FspI to SstI) from the 3′ end of the 347bp fragment further reduced its activity by 91% (Construct 4, Fig. 1), indicating that an important promoter element had been deleted.

The smallest fragment capable of generating detectable promoter activity (Construct 6, Fig. 2) is an 88bp sequence from SmaI at position −69bp to XbaI at position +19bp (Fig.3). As expected this activity was lost when its orientation was reversed (Construct 7, Fig. 2). To investigate whether the 59bp FspI−SstI fragment which spans the first exon/intron boundary could enhance the activity of this minimal promoter, a series of CAT reporter gene plasmids were constructed (Fig. 2). The 59bp fragment alone, when in either orientation, did not generate any CAT expression (Constructs 10 and 11, Fig. 2), however, when it was ligated in the correct and incorrect orientation with respect

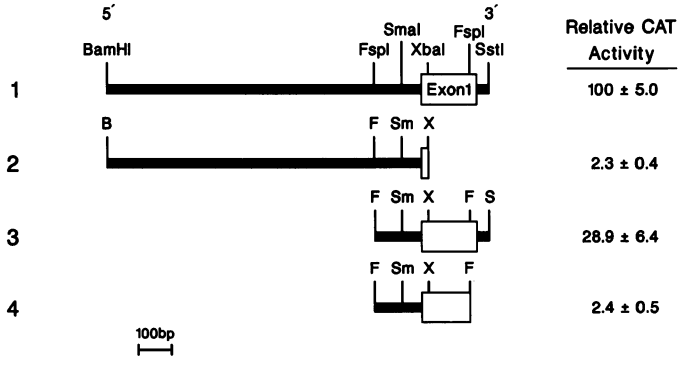

Figure 1. CAT activities generated by MGMT deletion fragments expressed as per cent of the 1.2kb maximal promoter fragment BamHI−SstI which is located at position −954 to +203 with respect to the transcription start site. Values represent the mean and standard deviations calculated from assays of at least three independent transfections.

to the SmaI−XbaI minimal promoter, CAT activity increased 9- and 13-fold respectively (Constructs 8 and 9, Fig. 2). The latter CAT activities were not significantly different from that produced by the entire 270bp SmaI−SstI MGMT promoter fragment which contains the 88bp SmaI−XbaI minimal promoter, the 59bp FspI−SstI fragment and 123bp of the intervening first exon sequence (Construct 5, Fig. 2).

The 59bp fragment enhancer function is not limited to the MGMT promoter or to one cell type

The 59bp FspI−SstI fragment was ligated in either orientation into a plasmid containing the UMP-synthase promoter and CAT reporter gene. These constructs, as well as CAT constructs containing either the MGMT minimal promoter alone, or together with the 59bp fragment in both orientations were independently transfected into the T lymphoblast cell line (CEM-CCRF) and the rhabdomyosarcoma cell line A204. The CAT:β-Gal ratios obtained are shown in Table I and the relative CAT activities are represented in Figure 4. In CEM-CCRF cells, the 59bp fragment increased the UMP-synthase promoter activity by 1.9- and 1.4-fold when it was in the correct and reversed orientation,

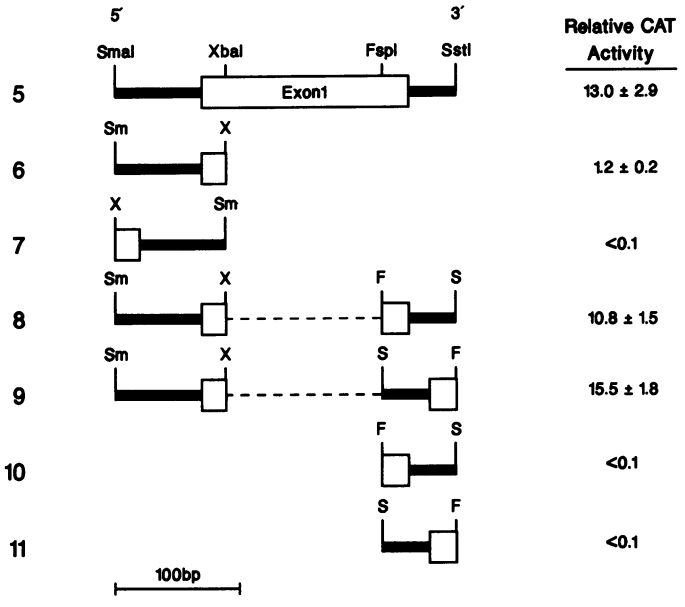

Figure 2. CAT activities (relative to the 1.2 kb maximal promoter = 100) generated by deletion fragments of a 273bp SmaI−SstI sequence located at position −69bp to +203bp with respect to the transcription start site. Values are relative to the maximal 1.2kb BamHI−SstI fragment shown in figure 1 and represent the mean and standard deviations from assays of at least three independent transfection experiments.

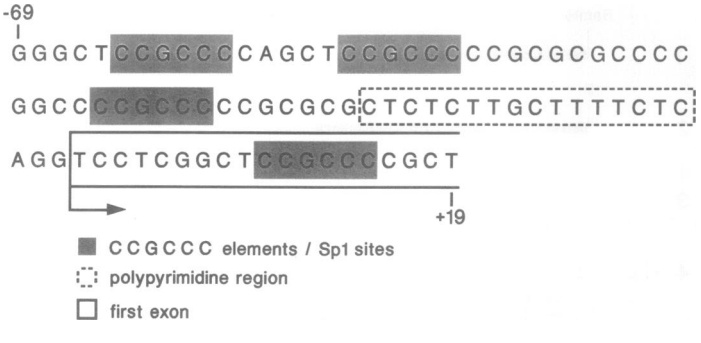

Figure 3. Sequence of the 88bp minimal promoter fragment with the position of the transcription start site indicated with the arrow (contained within EMBL accession No. X61657). The sequence representing position −69bp to +19bp with respect to the transcription start site is 80.7% GC rich. The area from position −19bp to −53bp is 100% GC.

Table I. Effect of 59bp enhancer fragment on MGMT and UMP-synthase promoter activities after transfection of CAT reporter gene constructs into CCRF-CEM and A204 cells

	Cell type CCRF-CEM	A204
MGMT promoter		
alone	27 ± 6[1]	0.72 ± 0.1
+ 59bp fragment	244 ± 34	6.13 ± 1.1
+ 59bp fragment reversed	350 ± 42	1.17 ± 0.1
UMP-synthase promoter		
alone	362 ± 32	4.50 ± 0.8
+ 59bp fragment	696 ± 71	25.6 ± 5.1
+ 59bp fragment reversed	501 ± 57	16.9 ± 1.9

[1]Normalized CAT activities ($\times 10^{-3}$) were calculated by dividing the CAT activity (cpm/h) by the β-galactosidase activity (A_{570}/h) after values for the promoterless control had been subtracted.

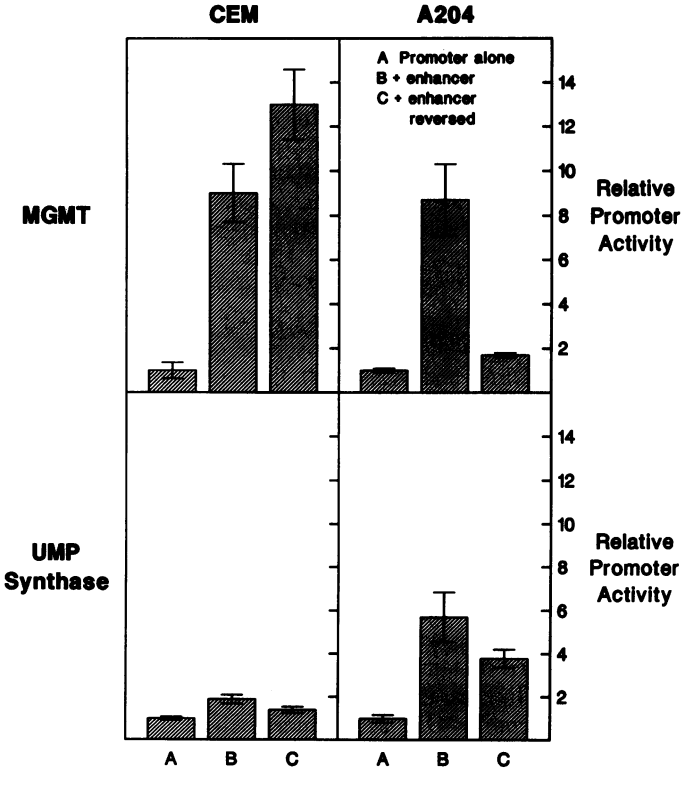

Figure 4. Relative MGMT and UMP-synthase promoter activities with and without the addition of the 59bp fragment in both orientations and in two cell lines. Values are relative to the basal level activity for each promoter which was designated as 1. Columns represent the mean of at least three experiments and the bars represent the standard deviations. **A**: Promoter alone; **B**: Promoter + enhancer; **C**: Promoter + enhancer in reverse orientation.

respectively. Following transfection of the same UMP-synthase promoter-CAT constructs into A204 cells, the 59bp fragment enhanced activity 5.7- and 3.8-fold, depending on orientation (Fig. 4). Similarly, with the MGMT promoter-CAT constructs, 8.7- and 1.7-fold increases in CAT expression were measured in A204 cells (Fig. 4).

In the absence of the 59bp enhancer, the MGMT minimal promoter was much less active than that of the UMP-synthase gene in either cell line, however, in CEM-CCRF cells after adding the enhancer, MGMT-CAT activity approached that produced by the UMP-synthase promoter (Table I).

The enhancer functions in 'cis' with respect to the promoter

To investigate whether the 59bp fragment could retain its enhancer function when transfected into cells in a plasmid separate from that containing the promoter, CAT activity was measured following transfection of UMP-synthase promoter CAT constructs into A204 cells. The activity generated by the UMP-synthase promoter-CAT construct (pCATUS2) when cotransfected with pUC59 (pUC18 containing the 59bp enhancer but lacking the CAT gene and promoter/enhancer elements) was no greater than the activity produced by the same amount of pCATUS2 cotransfected with pUC18 (Fig. 5). With the 59bp fragment inserted into the UMP-synthase construct (pCATUS259) CAT activity increased (Fig. 5) demonstrating that the enhancer is functional only when positioned in 'cis' with respect to a promoter.

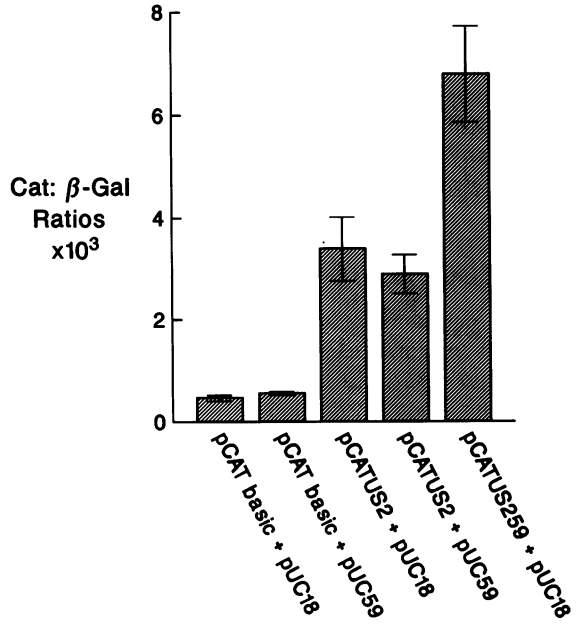

Figure 5. Histogram of CAT:β-Gal ratios demonstrating that the 59bp fragment will only function as an enhancer when placed in 'cis' with respect to the promoter. pUC18 does not contain the CAT gene or any promoter/enhancer elements; pUC59 is pUC18 containing the 59bp enhancer fragment; pCATbasic contains the CAT gene but no promoter/enhancer elements; pCATUS2 is pCATbasic containing the UMP-synthase promoter and pCATUS259 is pCATUS2 containing the 59bp enhancer fragment. Values were obtained by transfection of these constructs, together with pSVβ-Gal, into A204 cells and by assaying for CAT and β-Gal activity 48h posttransfection. Columns represent the mean of at least three experiments and the bars represent the standard deviations.

Enhancer-specific nuclear protein binding

Band mobility shift analysis using the 59bp enhancer fragment as a probe was carried out to investigate whether DNA binding proteins present within CEM-CCRF nuclear extract formed sequence-specific interactions. The 59bp FspI–SstI fragment was [^{32}P]-labeled and incubated with 3μg of CEM-CCRF nuclear extract together with various concentrations of competitor DNA as described in the methods. Figure 6 shows that in the presence of nuclear extract, all of the labeled probe was retarded during electrophoresis indicating that many (specific and nonspecific) DNA–protein complexes were formed (lane 2). The addition

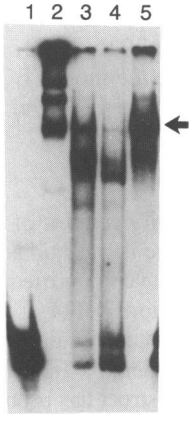

Figure 6. Band mobility shift analysis using the 59bp FspI–SstI fragment as a probe. Lane 1, ^{32}P-labeled 59bp probe alone; Lane 2, the probe in addition to 3μg of CCRF-CEM nuclear extract; Lanes 3 and 4, the contents of lane 2 with the addition of 5 and 10 fold molar excesses of unlabeled probe respectively; Lane 5, the contents of lane 2 with the addition of 10 fold molar excess of a nonspecific DNA fragment. The specific DNA–protein band is indicated with an arrow.

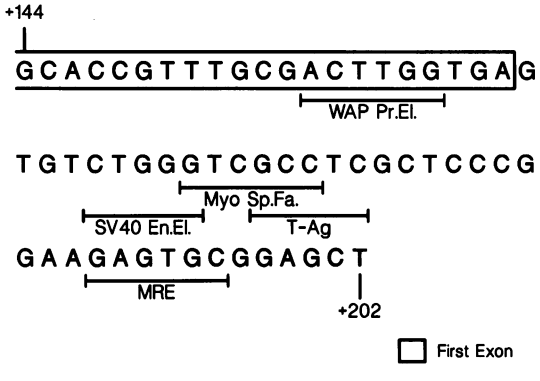

Figure 7. Sequence of the 59bp MGMT gene fragment including the complete restriction endonuclease recognition sequences for FspI (5′) and SstI (3′) (contained within EMBL accession no. X61657). FspI digests DNA between the C and G in the sequence TGCGCA and SstI cuts between the T and C in the sequence GAGCTC generating a 59bp fragment of DNA. The computer program 'Signal Scan' identified five matches to previously identified protein binding sites in other promoters; Myo Sp.Fa is the binding site for a myogenic specific factor present within the promoter of the actin gene (37), SV40 En.El. is a sequence known to be footprinted in the SV40 enhancer element (38), T-Ag is the core consensus sequence for the SV40 large T antigen binding site (39), MRE is a potential metal responsive element (40) and WAP Pr.El. is a sequence footprinted in the whey acidic protein gene promoter (41).

of 5- and 10-fold molar excess of unlabeled 59bp fragment (Fig. 6, lanes 3 and 4 respectively) resulted in the disappearance of one band presumably by competition for a specific binding protein. The specificity of this interaction was confirmed by the addition of an equivalent molar concentration of an irrelevant DNA fragment which did not compete for this protein binding (Fig. 6, lane 5).

The enhancer sequence was searched for known transcription factor binding motifs using the program 'Signal Scan' (36). Five regions with sequence homology to core elements of known binding sites were identified (Fig. 7), but their relevance to the 59bp enhancer function is unclear. Another potential protein binding site was identified by searching the GenBank/EMBL data base with the 59bp enhancer sequence using the Fasta program within the GCG software package (42). A 14bp region was identified from position +170 to +183, identical to nucleotides positioned at 399 to 412 in the mouse BCR gene promoter (43) except for one mismatch at +177/406.

DISCUSSION

During an attempt to identify regions of the MGMT regulatory sequence that are responsive to variations in transcription factors between cell types, we have identified a small (59bp) DNA fragment located at position +144 to +202 relative to the transcription start site, that is required for efficient promoter function. When this fragment is ligated to an 88bp minimal promoter fragment, the construct has promoter activity equivalent to that produced by the continuous 273bp SmaI−SstI sequence (Fig. 2) suggesting that the majority of the first exon does not contain important regulatory elements required for efficient promoter function. The 59bp fragment fits the general criteria for an 'enhancer' element based on the observations that its function is unaffected by position or orientation (44, Fig. 2)

The observation that transcription driven by the UMP-synthase promoter is enhanced by the 59bp fragment when in either orientation and also when transfected into another cell line (A204) (Fig.4) suggests that the enhancer may have a general function, not limited to the MGMT promoter or to CCRF-CEM cells. The relatively low 1.9 fold enhancement of UMP-synthase promoter activity in CCRF-CEM cells (Fig.4) can be explained by the high basal UMP-synthase promoter activity (Table I) such that if there is a maximum transcription rate (due to saturation of available transcription factors or limiting association/dissociation rates for the basal transcription machinery), the scope for enhancement would be limited. In contrast, the UMP-synthase promoter generates only low level basal activity in A204 cells, affording greater scope for enhancement (Fig. 4).

Whereas the enhancer functions efficiently in both orientations with the MGMT promoter in CCRF-CEM cells, in A204 cells its effect in the reverse orientation is relatively less (Fig. 4). The reverse orientation enhancer−UMP-synthase promoter construct is active in these cells suggesting that the required transacting factors are present. Hence, the apparent incompatibility of the MGMT promoter/reversed enhancer sequence and the regulatory proteins in A204 cells is surprising. One possible explanation for this orientation dependence is that the MGMT enhancer may require assembly of a three-dimensional nucleoprotein complex for activation (45), the formation of which could become disrupted under certain circumstances.

We excluded the possibility that the enhancer acts simply by competing for a suppressor binding protein by testing its function when transfected in 'trans' with respect to the promoter (Fig. 5). The requirement for the enhancer to be located on the same piece of DNA as the promoter (in 'cis') suggests that enhancer binding protein interacts with other proteins bound to the promoter region. The ability of this enhancer to function with two distinct promoters and in two separate cell lines also suggests that the enhancer binding protein interacts with common elements of the transcription machinery. Differential expression of any of these binding proteins may account for variation in MGMT transcription levels in Mer^+ cells.

Evidence for a DNA binding protein specific for the 59bp enhancer sequence was obtained from the band shift experiment shown in Figure 6. There are several consensus sequences in the 59bp fragment that match previously published binding motifs (Fig. 7). However, the myocyte specific factor and the SV40 large T antigen are probably not relevant for MGMT expression as they are not expressed in CCRF-CEM cells. The metal responsive element is also unlikely to be involved because the human MGMT promoter is not inducible by metal ions (data not shown). The remaining two consensus sequences previously identified by footprinting studies of the whey acidic protein promoter and the SV40 enhancer, could be potential regulatory sites in the 59bp enhancer sequence, however, their binding proteins have yet to be identified. The 'Signal Scan' program used to identify these sites acknowledges that it finds more erroneous motifs than significant ones but that statistical significance increases with sequence length. The motifs identified in the 59bp enhancer are only five and six nucleotides long and therefore have a relatively high probability of random occurrence. The 14bp region of homology between the mouse BCR gene promoter and the 59bp enhancer is of interest, however, it is not known if any proteins bind this site, moreover, this sequence is not present in any human DNA cloned to date. There are thus no clear candidates for proteins that bind this 59bp enhancer, hence, it is possible that a novel trans-acting factor is involved. We are currently investigating the precise protein binding sites in the 59bp enhancer region by DNase I footprinting.

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